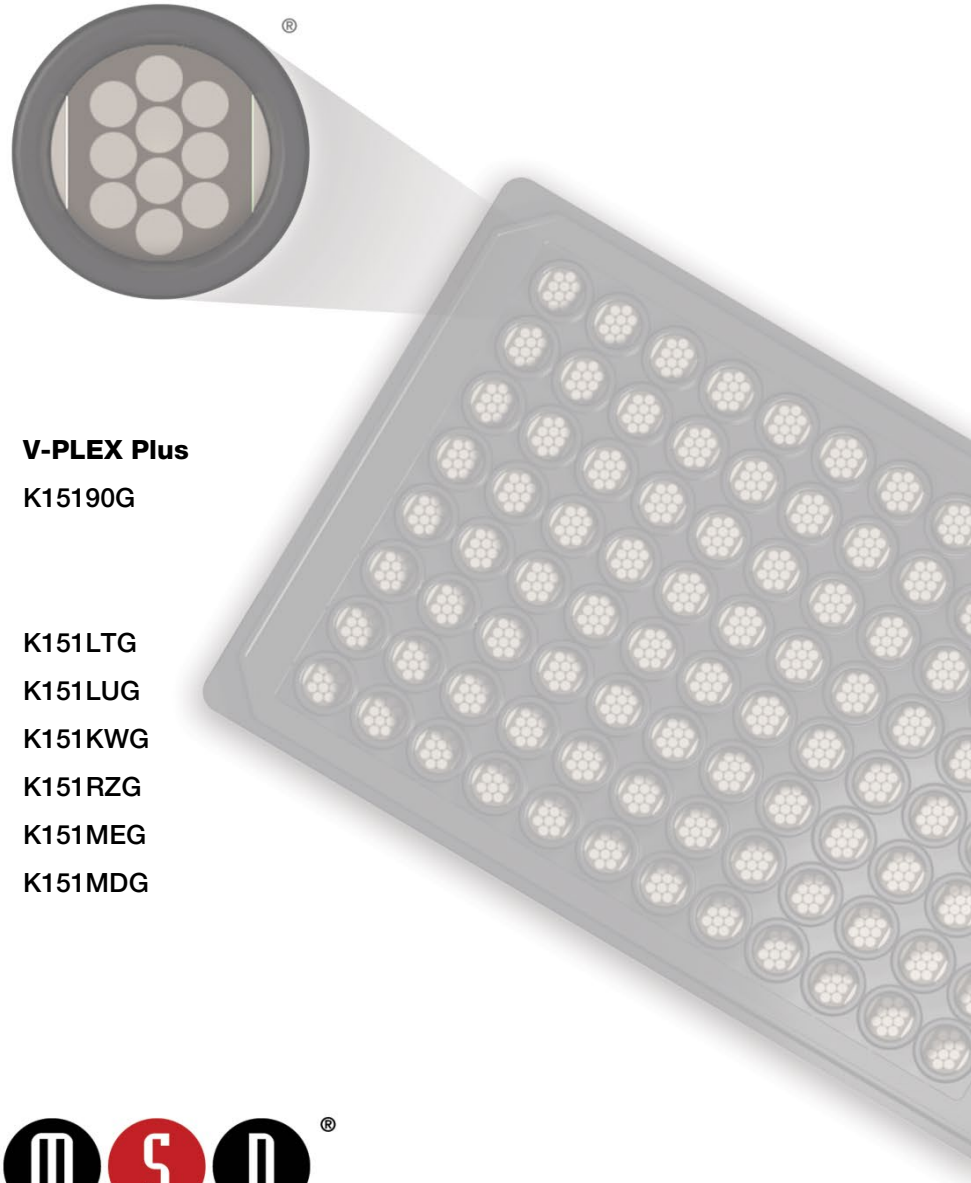
Angiogenesis Panel 1 (human) Kits

VEGF-A, VEGF-C, VEGF-D, Tie-2, Flt-1, PlGF, FGF (basic)

V-PLEX®



	V-PLEX®	V-PLEX Plus
Multiplex Kits	K15190D	K15190G
Individual Assay Kits		
Human VEGF-C	K151LTD	K151LTG
Human VEGF-D	K151LUD	K151LUG
Human Tie-2	K151KWD	K151KWG
Human Flt-1	K151RZD	K151RZG
Human PlGF	K151MED	K151MEG
Human FGF (basic)	K151MDD	K151MDG

MSD Biomarker Assay

Angiogenesis Panel 1 (human) Kits

VEGF-A, VEGF-C, VEGF-D, Tie-2, Flt-1, PlGF, and FGF (basic)

For use with serum, EDTA plasma, cell culture supernatant, urine, cerebral spinal fluid, and cell lysate.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®
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Table of Contents

Introduction 4
Principle of the Assay 5
Kit Components 6
Additional Materials and Equipment 8
Optional Materials and Equipment 8
Safety 8
Best Practices 9
Reagent Preparation 10
Assay Protocol 13
Validation 14
Analysis of Results 16
Typical Data 16
Sensitivity 17
Precision 18
Dilutional Linearity 19
Spike Recovery 20
Specificity 20
Species Cross-Reactivity 21
Stability 21
Calibration 21
Tested Samples 22
Assay Components 23
References 23
Appendix A 24
Summary Protocol 25
Catalog Numbers 26
Plate Layout 27
Plate Diagram 28

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Introduction

MSD offers V-PLEX assays for customers who require unsurpassed performance and quality. V-PLEX products are developed under rigorous design control and are fully validated according to fit-for-purpose principles[1] following MSD's Quality Management System. They offer exceptional sensitivity, simple protocols, reproducible results, and lot-to-lot consistency. In addition to the analytical validation, the robustness of the assay protocol is assessed during development along with the stability and robustness of the assay components and kits. V-PLEX assays are available in both single-assay and multiplex formats.

The V-PLEX assay menu is organized by panels. Grouping the assays into panels by species, analytical compatibility, clinical range, and expected use ensures optimal and consistent performance from each assay while still providing the benefits and efficiencies of multiplexing. V-PLEX panels are provided in MSD's MULTI-SPOT® 96-well plate format. The composition of the panel and the location of each assay (i.e., its spot within the well) are maintained from lot to lot. Most individual V-PLEX assays are provided on MSD's small spot plates. The remaining are provided on the multiplex panel plate.

The Angiogenesis Panel 1 (human) measures seven biomarkers that are proangiogenic proteins: VEGF-A, VEGF-C, VEGF-D, Tie-2, Flt-1 (sFlt-1/VEGF-R1), PlGF, and FGF (basic) previously known as bFGF. Angiogenesis is essential to the process of neovascularization under both physiological conditions and pathological conditions, including cancer, diabetic retinopathy, rheumatoid arthritis, and ischemia. Angiogenesis is modulated by initiatory and inhibitory molecules. Research efforts on tumor angiogenesis involve targeting pathways of these biomarkers as well as targeting their receptors. For example, several different strategies have been designed to target vascular endothelial growth factor receptor (Flt-1) signal transduction. Also, several VEGF-neutralizing antibodies have been successful in combination with chemotherapy in limiting angiogenesis and tumor progression. Other strategies to target the VEGF/Flt-1 pathway include soluble VEGFRs (traps), receptor tyrosine-kinase inhibitors that target VEGFR2/Flk-1, and neutralizing aptamers. A better understanding of signaling pathways in tumor angiogenesis, in addition to VEGF receptor signaling, may help in advancing this research on anticancer therapy. This product insert describes a multiplex panel of key biomarkers that may be useful in angiogenesis research. The biomarkers constituting the Angiogenesis Panel 1 (human) Kits are: **a)** vascular endothelial growth factor-A (VEGF or VEGF-A), **b)** vascular endothelial growth factor-C (VEGF-C), **c)** vascular endothelial growth factor-D (VEGF-D), **d)** tyrosine kinase-2 (Tie-2), **e)** Flt-1 (fms-like tyrosine kinase 1 or sFlt-1/VEGF-R1), **f)** placental growth factor (PlGF), and **g)** basic fibroblast growth factor (FGF (basic)).

Principle of the Assay

MSD biomarker assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the Angiogenesis Panel 1 (human) are sandwich immunoassays. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots, as shown in the layouts below. Multiplex assays and the individual VEGF-C, VEGF-D, Tie-2, Flt-1, PlGF, and FGF (basic) assays are provided on 7-spot MULTI-SPOT plates (Figure 1); the individual VEGF-A assay is provided on Small Spot plates (Figure 2). The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) throughout one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that creates the appropriate chemical environment for electrochemiluminescence (ECL) and loads the plate into an MSD® instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample. V-PLEX assay kits have been validated according to the principles outlined in "Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement" by J. W. Lee, et al.[1]

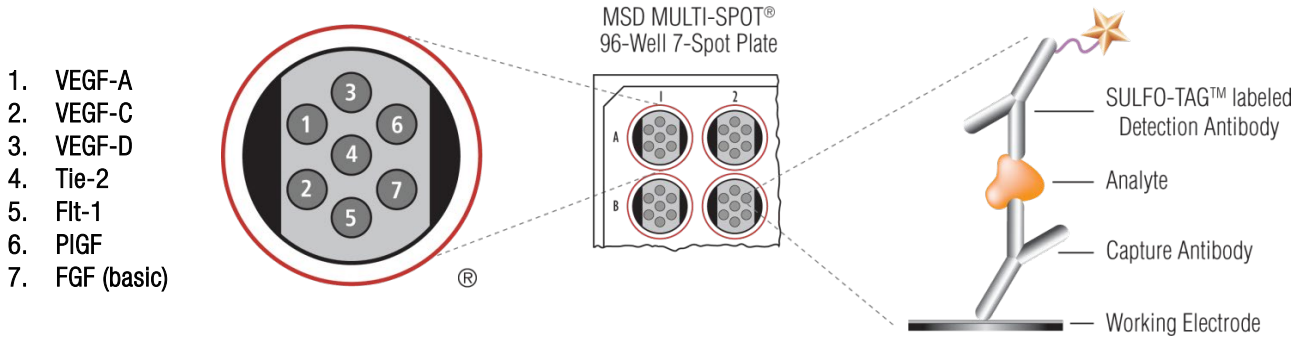

Figure 1. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

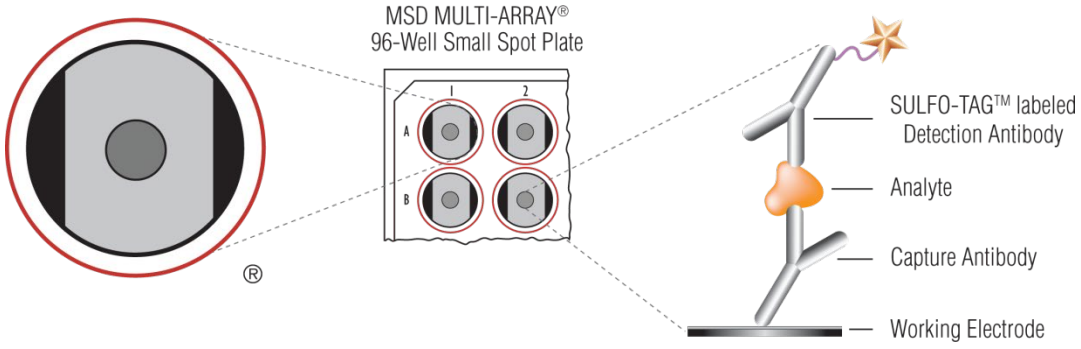

Figure 2. Small Spot plate diagram showing placement of analyte capture antibodies.

Kit Components

Angiogenesis Panel 1 (human) Assays are available as a 7-spot multiplex kit, as individual assay kits, or as custom V-PLEX kits with subsets of assays selected from the full panel. V-PLEX Plus kits include additional items (controls, wash buffer, and plate seals). See below for details.

See the **Catalog Numbers** section for complete kits.

Reagents Supplied with All Kits

Table 1. Reagents that are supplied with V-PLEX and V-PLEX Plus Kits

Reagent	Storage	Catalog No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Angiogenesis Panel 1 (human) Calibrator Blend (20X)*	≤–70 °C	C0666-2	60 µL	1 vial	5 vials	25 vials	Calibrator blend; individual analyte concentration is provided in the lot-specific certificate of analysis (COA).
Diluent 7	≤–10 °C	R54BB-3	50 mL	1 bottle	1 bottle	5 bottles	Diluent for samples and calibrator.
Diluent 11	≤–10 °C	R55BA-4	5 mL	1 bottle			Diluent for detection antibody.
		R55BA-3	50 mL		1 bottle	5 bottles	
Blocker A Kit	RT	R93AA-2	250 mL	1 kit	1 kit	5 kits	Blocking solution kit.
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	Buffer to catalyze the electro-chemiluminescent reaction.

*The calibrator blend (catalog No. C0190-2) was discontinued and replaced by a calibrator blend containing new recombinant VEGF-C protein (catalog No. C0666-2).

V-PLEX Plus Kits: Additional Components

Table 2. Additional components that are supplied with V-PLEX Plus Kits

Reagents	Storage	Catalog No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Angiogenesis Control 1*	≤–70 °C	—	1 vial	1 vial	5 vials	25 vials	Multi-analyte controls. The concentration of the controls is provided in the lot-specific COA.
Angiogenesis Control 2*	≤–70 °C		1 vial	1 vial	5 vials	25 vials	
Angiogenesis Control 3*	≤-70 °C		1 vial	1 vial	5 vials	25 vials	
Wash Buffer (20X)	RT	R61AA-1	100 mL	1 bottle	5 bottles	25 bottles	20-fold concentrated plate wash buffer solution.
Plate Seals	—	—	—	3	15	75	Adhesive seals for sealing plates during incubations.

*Controls are also provided as components in the Angiogenesis Control Pack 1 (catalog# C4666-1, 5-plate size pack). Refer to the control pack insert for more details. **Note**: The control pack (catalog No. C4190-1) was discontinued and replaced by a control pack containing new recombinant VEGF-C protein (catalog No. C4666-1).

Kit-Specific Components

Table 3. Components that are supplied with specific kits

Plates	Storage	Part No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Angiogenesis Panel 1 (human) Plate*	2–8 °C	N75666A-1	7-spot	1	5	25	96-well plate, foil sealed, with desiccant.

*The Angiogenesis panel 1 (human) plate (catalog No. N75190A-1) was discontinued and replaced by an Angiogenesis Panel 1 (human) plate containing new monoclonal capture antibody for the VEGF-C assay (catalog No. N75666A-1).

Table 4. Individual detection antibodies for each assay are supplied with specific kits

SULFO-TAG Detection Antibody	Storage	Catalog No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Anti-hu VEGF-A Antibody (50X)	2–8 °C	D21KL-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21KL-3	375 µL	-	1	5	
Anti-hu VEGF-C Antibody (50X)*	2–8 °C	D21AKY-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21AKY-3	375 µL	-	1	5	
Anti-hu VEGF-D Antibody (50X)	2–8 °C	D21LU-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21LU-3	375 µL	-	1	5	
Anti-hu Tie-2 Antibody (50X)	2–8 °C	D21KW-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21KW-3	375 µL	-	1	5	
Anti-hu Flt-1 Antibody (50X)	2–8 °C	D21EJ-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21EJ-3	375 µL	-	1	5	
Anti-hu PlGF Antibody (50X)	2–8 °C	D21ME-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21ME-3	375 µL	-	1	5	
Anti-hu FGF (basic) Antibody (50X)	2–8 °C	D21MD-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21MD-3	375 µL	-	1	5	

*The anti-human VEGF-C antibody (catalog No. D21LT) was discontinued and replaced by a new monoclonal detection antibody for the VEGF-C assay (catalog No. D21AKY).

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing dilutions
- ❑ Liquid-handling equipment for the desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
- ❑ Plate-washing equipment: automated plate washer or multichannel pipette
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500–1,000 rpm
- ❑ Phosphate-buffered saline (PBS) plus 0.05% Tween-20 for plate washing or MSD Wash Buffer catalog no. R61AA-1 (included in V-PLEX Plus kit)
- ❑ Adhesive plate seals (3 per plate included in V-PLEX Plus kits)
- ❑ Deionized water
- ❑ Vortex mixer

Optional Materials and Equipment

- ❑ Angiogenesis Control Pack 1, available for separate purchase from MSD, catalog no. C4666-1 (included in V-PLEX Plus kit)
- ❑ Centrifuge for sample preparation

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the applicable safety data sheet(s) (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

Best Practices

- Do not mix or substitute reagents from different sources or different kit lots. Lot information is provided in the lot-specific COA.
- Assay incubation steps should be performed at 20 °C to 26 °C to achieve the most consistent signals between runs.
- Bring frozen diluent to room temperature in a 24 °C water bath. Thaw other reagents on wet ice and use them as directed immediately.
- Prepare calibrators, samples, and controls in polypropylene microcentrifuge tubes. Use a fresh pipette tip for each dilution, and vortex after each dilution before proceeding.
- Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD plate.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding Read Buffer T may interfere with signal detection.
- Use reverse pipetting when necessary to avoid the introduction of bubbles. For empty wells, pipette gently to the bottom corner.
- Plate shaking should be vigorous, with a rotary motion between 500 and 1,000 rpm. Binding reactions may reach equilibrium sooner if you use shaking at the middle of this range (~700 rpm) or above.
- When using an automated plate washer, rotate the plate 180 degrees between wash steps to improve assay precision.
- Washing plates with a high volume of wash buffer, 3 times with 300 µL/well, may provide improvement in assay precision for certain assays without impacting the analytical parameters, including LOQs, control recovery, and sample quantification.
- Tap the plate on a paper towel to remove residual fluid after washing.
- Read buffer should be at room temperature when added to the plate.
- If an incubation step needs to be extended, leave the sample or detection antibody solution in the plate to keep the plate from drying out.
- Remove the plate seal before reading the plate.
- Do not shake the plate after adding Read Buffer T.
- Keep time intervals consistent between adding read buffer and reading the plate to improve inter-plate precision. Unless otherwise directed, read the plate as soon as possible after adding read buffer.
- If assay results are above the top of the calibration curve, dilute the samples and repeat the assay.
- When running a partial plate, seal the unused sectors to avoid contaminating unused wells. Remove all seals before reading. Partially used plates may be sealed and stored for up to 30 days at 2–8 °C in the original foil pouch with desiccant. You may adjust volumes proportionally when preparing reagents.

Reagent Preparation

Bring all diluents and buffers to room temperature.

Important: Upon the first thaw, aliquot Diluent 7 and Diluent 11 into suitable volumes before refreezing.

Prepare Blocker A Solution

Follow the preparation procedure in the product insert (also available at www.mesoscale.com) provided with the Blocker A Kit to prepare the Blocker A solution.

For one plate use, follow steps indicated below:

- ❑ Weigh out 1.25 g of Blocker A dry powder.
- ❑ Add 20 mL of deionized water. Stir until all protein is resuspended.
- ❑ Add 5 mL of MSD Phosphate Buffer (5X).

Prepare Calibrator Dilutions

MSD supplies a multi-analyte calibrator that yields the recommended highest calibrator concentration when diluted 20-fold in Diluent 7. (For individual assays that do not saturate at the highest calibrator concentration, the calibration curve can be extended by creating a more concentrated highest calibrator. In that case, follow the steps below using a dilution factor <20 when diluting the stock calibrator.)

To prepare 7 calibrator solutions plus a zero calibrator for up to 4 replicates:

- ❑ Thaw the stock calibrator on wet ice for at least 30 minutes and keep on ice.
- ❑ Prepare the highest calibrator by adding 20 µL of stock calibrator to 380 µL of Diluent 7. Mix well by vortexing.
- ❑ Prepare the next calibrator by transferring 100 µL of the highest calibrator to 300 µL of Diluent 7. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators. Diluted calibrator may be stored on wet ice for up to 60 minutes before use.

Important: Use Diluent 7 as the zero calibrator.

Note: Stock calibrator may be stored at ≤–70 °C and may be refrozen and thawed once. For the lot-specific concentration of each calibrator in the blend, refer to the COA supplied with the kit. You can also find a copy of the COA at www.mesoscale.com.

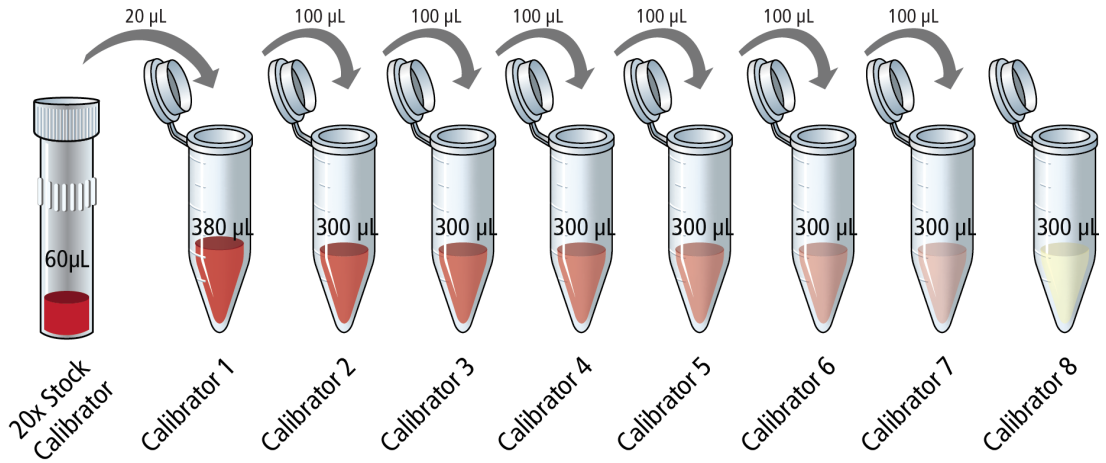

Figure 3. Dilution schema for preparation of Calibrator Standards.

Sample Collection and Handling

Below are general guidelines for human sample collection, storage, and handling. If possible, use published guidelines.[2] Evaluate sample stability under the selected method as needed.

- Serum and plasma. When preparing serum, allow samples to clot for 2 hours at room temperature, then centrifuge for 20 minutes at 2,000 × *g* before using or freezing. If no particulates are visible, you may not need to centrifuge.
- Other samples. Use immediately or freeze.

Freeze all samples in suitably sized aliquots; they may be stored at ≤-10 ºC until needed. Repeated freeze-thaw of samples is not recommended. After thawing, centrifuge samples at 2,000 × *g* for 3 minutes to remove particulates before sample preparation.

Dilute Samples

Dilute samples with Diluent 7. For human serum and EDTA plasma samples, MSD recommends a minimum 2-fold dilution. For example, when running samples in duplicate, add 60 µL of sample to 60 µL of Diluent 7. We recommend running at least two replicates per sample. When running unreplicated samples, add 40 µL of sample to 40 µL of Diluent 7. You may conserve sample volume by using a higher dilution. Tissue culture supernatants may require additional dilution based on stimulation and analyte concentrations in the sample. The kit includes diluent sufficient for running samples in duplicate. Additional diluent can be purchased at www.mesoscale.com.

Prepare Controls

Three levels of multi-analyte controls are available for separate purchase from MSD in the Angiogenesis Control Pack, catalog no. C4666-1. (Controls are included in V-PLEX Plus kits.) Controls 1, 2, and 3 are prepared in a non-matrix-based diluent; all controls are spiked with recombinant human analytes.

Thaw the controls on wet ice for at least 30 minutes. Dilute controls 2-fold in Diluent 7. Discard unused diluted control material.

Undiluted controls are stored at ≤–70 ºC and can tolerate three freeze-thaw cycles. For the lot-specific concentration of each analyte in the control, refer to the Angiogenesis Control Pack 1 COA.

Prepare Detection Antibody Solution

MSD provides each detection antibody separately as a 50X stock solution. The working solution is 1X. Prepare the detection antibody solution immediately before use.

7-plex Angiogenesis Panel 1 (human) kit

For one plate, combine the following detection antibodies and add to 2,580 µL of Diluent 11:

- ❑ 60 µL of SULFO-TAG Anti-hu VEGF-A Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu VEGF-C Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu VEGF-D Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu Tie-2 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu Flt-1 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu PlGF Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu FGF (basic) Antibody

Custom multiplex kits

For one plate, combine 60 µL of each supplied detection antibody, then add Diluent 11 to bring the final volume to 3,000 µL.

Individual assay kits

For one plate, add 60 µL of the supplied detection antibody to 2,940 µL of Diluent 11.

Prepare Wash Buffer

MSD provides 100 mL of Wash Buffer as a 20X stock solution in the V-PLEX Plus kit. Dilute the stock solution to 1X before use. PBS + 0.05% Tween-20 can be used instead.

For one plate, combine:

- ❑ 15 mL of Wash Buffer (20X)
- ❑ 285 mL of deionized water

Prepare Read Buffer T

MSD provides Read Buffer T as a 4X stock solution. The working solution is 2X.

For one plate, combine:

- ❑ 10 mL of Read Buffer T (4X)
- ❑ 10 mL of deionized water

You may keep excess diluted Read Buffer T in a tightly sealed container at room temperature for up to one month.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates may be used as delivered; no additional preparation is required.

Assay Protocol

Note: Follow **Reagent Preparation** before beginning this assay protocol.

STEP 1: Add Blocker A Solution

- ❑ Add 150 µL of Blocker A solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 1 hour.

STEP 2: Wash and Add Sample

- ❑ Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 50 µL of diluted sample, calibrator, or control per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Add Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

STEP 4: Wash and Read

- ❑ Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 150 µL of 2X Read Buffer T to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer T is not required before reading the plate.

Alternate Protocol

The suggestion below may be useful as an alternate protocol; however, it was not tested using multiple kit lots.

Alternate Protocol, Dilute-in-Plate: To limit sample handling, you may dilute samples and controls in the plate. For 2-fold dilution, add 25 µL of assay diluent to each sample/control well, and then add 25 µL of neat control or sample. Calibrators should not be diluted in the plate; add 50 µL of each calibrator solution directly into empty wells. Tests conducted according to this alternate protocol produced results that were similar to the recommended protocol (data not shown).

Validation

V-PLEX products are validated following fit-for-purpose principles[1] and MSD design control procedures. V-PLEX assay components go through an extensive critical reagents program to ensure that the reagents are controlled and well characterized. Before the release of each V-PLEX panel, at least three independent kit lots are produced. Using results from multiple runs (typically greater than 50) and multiple operators, these lots are used to establish production specifications for sensitivity, specificity, accuracy, and precision. During validation, each assay is analytically validated as a singleplex and is also independently evaluated as a multiplex component by running the full multiplex plate using only the single detection antibody for that assay. These results are compared with the results from the multiplex panel when using all detection antibodies. This demonstrates that each assay is specific and independent, allowing them to be multiplexed in any combination. The COA provided with each kit outlines the kit release specifications for sensitivity, specificity, accuracy, and precision.

- ### Dynamic Range

  Calibration curve concentrations for each assay are optimized for a maximum dynamic range while maintaining enough calibration points near the bottom of the curve to ensure a proper fit for accurate quantification of samples that require high sensitivity.

- ### Sensitivity

  The lower limit of detection (LLOD) is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The LLOD is calculated using results from multiple plates for each lot, and the median and range of calculated LLODs are reported in this product insert. The upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) are established for each lot by measuring multiple levels near the expected LLOQ and ULOQ levels. The final LLOQ and ULOQ specifications for the product are established after the assessment of all validation lots.

- ### Accuracy and Precision

  Accuracy and precision are evaluated by measuring calibrators and matrix-based validation samples or controls across multiple runs and multiple lots. For most assays, the results of control measurements fall within 25% of the expected concentration for each run. Precision is reported as the coefficient of variation (CV). Intra-run CVs are typically below 5%, and inter-run CVs are typically below 15%. Rigorous management of inter-lot reagent consistency and calibrator production results in typical inter-lot CVs of less than 25%. Validation lots are compared using controls and at least 20 samples in various sample matrices. Samples are well correlated with an inter-lot bias typically below 10%.

- ### Matrix Effects and Samples

  Matrix effects from serum, EDTA plasma, and cell culture media are measured as part of development and validation. Dilutional linearity and spike recovery studies are performed on individual samples rather than pooled samples to assess the variability of results due to matrix effects. The sample dilution suggested in the protocol gives an appropriate dilution factor for all assays in the multiplex. Some assays may benefit from higher dilution factors, depending on the samples and application (data is provided in the product insert). In addition to the matrices listed above, urine, CSF, and cell lysates samples were assayed, but dilutional linearity and spike recovery studies were not performed.

- **Specificity**

  The specificity of both capture and detection antibodies is measured during assay development. Antibody specificity is assessed by first running each assay using the multiplex plate with assay-specific detection antibody and assay-specific calibrator. These results are compared to the assay's performance when the plate is run with the multi-analyte calibrator and assay-specific detection antibodies and with assay-specific calibrator and all detection antibodies. For each validation lot and product release, assay specificity is measured using a multi-analyte calibrator and individual detection antibodies. The calibrator concentration used for specificity testing is chosen to ensure that the specific signal is greater than 50,000 counts.

  In addition to measuring the specificity of antibodies to analytes in the multiplex kit, specificity, and interference from other related markers are tested during development. This includes the evaluation of selected related proteins and receptors or binding partners.

- **Assay Robustness and Stability**

  The robustness of the assay protocol is assessed by examining the boundaries of the selected incubation times and evaluating the stability of assay components during the experiment and storage. For example, assay component (calibrator, antibody, control) stability was assessed through freeze-thaw testing and accelerated stability studies. The validation program includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

Representative data from the validation studies are presented in the following sections. The calibration curve and measured limits of detection for each lot can be found in the lot-specific COA that is included with each kit and available for download at www.mesoscale.com.

Analysis of Results

The calibration curves used to calculate analyte concentrations were established by fitting the signals from the calibrators to a 4-parameter logistic (or sigmoidal dose-response) model with a $1/Y^2$ weighting. The weighting function provides a better fit of data over a wide dynamic range, particularly at the low end of the calibration curve. Analyte concentrations were determined from the ECL signals by back-fitting to the calibration curve. These assays have a wide dynamic range (4 logs), which allows accurate quantification of samples without the need for multiple dilutions or repeated testing. The calculations to establish calibration curves and determine concentrations were carried out using the MSD DISCOVERY WORKBENCH® analysis software.

The best quantification of unknown samples will be achieved by generating a calibration curve for each plate using a minimum of two replicates at each calibrator level.

Typical Data

Data from the Angiogenesis Panel 1 (human) were collected over 1 month of testing by three operators (47 runs in total, 20 runs for VEGF-C). Calibration curve accuracy and precision were assessed for three kit lots. Representative data from one lot are presented below. Calibration curves for each lot are presented in the lot-specific COA.

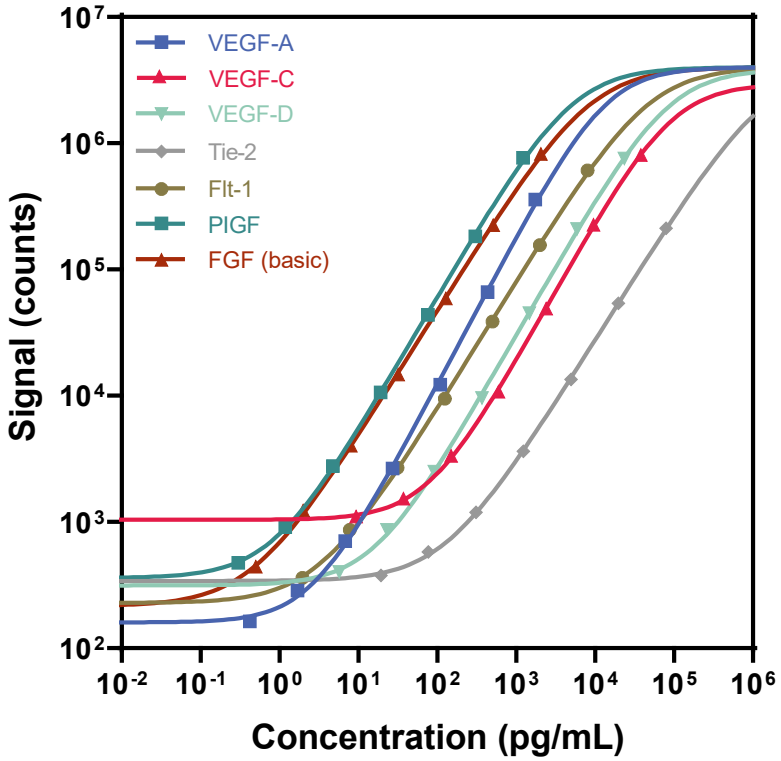

Figure 4. Typical calibration curves for the Angiogenesis Panel 1 (human) Assay

Additional data are available in the Appendix. **Appendix A** compares results for each assay in the kit when the panel is run using the individual detection antibody versus all seven detection antibodies. The calibration curves were comparable.

Sensitivity

The LLOD is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator). The LLOD shown below was calculated based on 47 runs (N = 20 runs for VEGF-C).

The ULOQ is the highest concentration at which the CV of the calculated concentration is <20% (<25% for PlGF) and the recovery of each calibrator is within 80% to 120% of the known value.

The LLOQ is the lowest concentration at which the CV of the calculated concentration is <20% (<25% for Flt-1, PlGF, and FGF (basic)), and the recovery of each calibrator is within 80% to 120% of the known value.

The quantitative range of the assay lies between the LLOQ and ULOQ.

The LLOQ and ULOQ are verified for each kit lot and the results are provided in the lot-specific COA that is included with each kit and available at www.mesoscale.com.

Table 5. LLOD, LLOQ, and ULOQ for each analyte in the Angiogenesis Panel 1 (human) Kit

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
VEGF-A	0.40	0.14–2.20	5.00	1,510
VEGF-C	10.5	7.11–17.5	146	17,500
VEGF-D	4.36	1.83–70.6	67.1	18,800
Tie-2	31.3	16.4–62.6	396	63,400
Flt-1	0.90	0.65–1.21	10.0	6,410
PlGF	0.21	0.04–1.42	1.50	800
FGF (basic)	0.09	0.07–0.22	2.60	1,780

Precision

Controls were made by spiking calibrator into a non-human matrix at three levels within the quantitative range of the assay. Analyte levels were measured by three operators using a minimum of three replicates on 46 runs (N = 20 for VEGF-C) over 1 month. Results are shown below. Both intra- and inter-day run precision are CVs of less than 15% for this panel.

Average intra-run % CV is the average % CV of the control replicates within an individual run.

Inter-run % CV is the variability of controls across 46 runs.

Inter-lot % CV is the variability of controls across three kit lots.

Table 6. Intra-run and Inter-run % CVs for each analyte in the Angiogenesis Panel 1 (human) Kit

	Control	Average Conc. (pg/mL)	Average Intra-run % CV	Inter-run % CV	Inter-lot % CV
VEGF-A	Control 1	1,535	1.9	4.7	2.1
	Control 2	129	2.1	6.3	3.8
	Control 3	16.7	2.7	6.0	2.8
VEGF-C	Control 1	6,677	6.0	9.2	2.5
	Control 2	2,666	6.2	9.2	2.9
	Control 3	1,064	7.0	9.6	2.7
VEGF-D	Control 1	22,280	4.7	5.4	0.4
	Control 2	3,562	4.4	6.3	1.5
	Control 3	654	3.9	7.1	0.2
Tie-2	Control 1	68,434	4.9	5.3	2.2
	Control 2	9,475	5.7	8.9	6.8
	Control 3	1,721	4.0	9.2	4.9
Flt-1	Control 1	7,422	1.7	5.2	3.6
	Control 2	620	2.4	6.1	5.1
	Control 3	71.7	3.5	7.3	2.7
PlGF	Control 2	205	2.1	7.8	6.3
	Control 3	22.3	2.3	7.7	5.6
FGF (basic)	Control 1	1,559	3.4	4.6	1.3
	Control 2	137	3.5	5.8	0.7
	Control 3	16.8	4.2	6.9	1.6

Dilutional Linearity

To assess linearity, normal human serum and EDTA plasma from a commercial source as well as cell culture supernatants were spiked with recombinant calibrators and diluted 2-fold, 4-fold, 8-fold, and 16-fold before testing. Percent recovery at each dilution was calculated by dividing the dilution-adjusted concentration by the expected concentration, i.e., the dilution-adjusted concentration at 2-fold dilution. The average percent recovery shown below is based on samples within the quantitative range of the assay.

$$\%\, recovery = \frac{measured\ concentration}{expected\ concentration} \times 100$$

Table 7. Analyte percent recovery at 4-fold, 8-fold, and 16-fold dilutions of each sample type

		VEGF-A		VEGF-C*		VEGF-D		Tie-2	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N = 5)	4	113	99–136	85	81–92	111	101–126	117	105–127
	8	116	108–137	71	68–77	122	103–149	134	112–151
	16	114	108–133	68	65–71	121	105–133	121	93–143
EDTA Plasma (N = 5)	4	109	103–113	76	71–85	115	100–138	103	101–105
	8	109	103–118	69	60–79	130	106–198	104	92–121
	16	103	94–114	69	59–77	147	108–266	104	82–129
Cell Culture Supernatant (N = 3)	4	101	95–108	87	86–88	106	104–108	94	94–95
	8	98	88–115	78	74–83	103	100–109	88	86–90
	16	93	79–112	70	69–72	94	91–99	80	79–82

*VEGF-C was testing using N = 3 for serum and EDTA plasma, and N = 2 for cell culture supernatants.

		Flt-1		PlGF		FGF (basic)	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N = 5)	4	113	100–123	103	101–106	97	86–112
	8	118	113–134	101	98–104	94	87–102
	16	115	106–127	103	99–106	98	90–104
EDTA Plasma (N = 5)	4	105	93–118	108	103–113	95	73–103
	8	114	103–131	105	99–112	95	82–107
	16	117	100–139	109	105–118	95	92–102
Cell Culture Supernatant (N = 3)	4	111	97–124	92	88–96	72	69–78
	8	110	95–126	89	86–93	70	65–80
	16	102	91–111	87	80–92	67	63–75

Spike Recovery

Spike and recovery measurements of different sample types across the quantitative range of the assays were evaluated. Multiple individual human sera and EDTA plasma samples from a commercial source as well as cell culture supernatants were spiked with calibrators at three levels (high, mid, and low) and then diluted 2-fold. The average percent recovery for each sample type is reported along with % CV and percent recovery range.

$$\%\ recovery = \frac{measured\ concentration}{expected\ concentration} \times 100$$

Table 8. Spike and Recovery measurements of different sample types evaluated in the Angiogenesis Panel 1 (human) Kit

	Serum (N = 5)			EDTA Plasma (N = 5)			Cell Culture Supernatants[4] (N = 3)		
	Average % Recovery	% CV	% Recovery Range	Average % Recovery	% CV	% Recovery Range	Average % Recovery	% CV	% Recovery Range
VEGF-A	87	5.4	80–97	110	22.0	94–193[1]	117	18.7	66–132
VEGF-C*	144	4.1	114–165	124	3.1	90–169	138	3	113–174
VEGF-D	100	16.3	71–130	104	8.0	92–120	118	9.4	99–132
Tie-2[2]	53	21.4	32–69	61	40.9	24–113	120	27.6	90–196
Flt-1[3]	68	36.9	28–99	95	9.6	72–110	92	14.7	68–109
PlGF	103	6.1	93–114	103	7.2	92–114	107	3.1	102–111
FGF (basic)	89	11.2	71–113	83	36.0	35–145	132	24.4	97–182

[1] VEGF-A over-recovery was observed in one EDTA plasma sample spiked with a low level of recombinant VEGF-A.
[2] Tie-2 under-recovery was observed at all spike levels in serum and EDTA plasma. This may be attributed to binding of the recombinant protein to endogenous Ang-1 and Ang-2 ligands.
[3] Flt-1 under-recovery was observed in all serum samples spiked with the high level of recombinant Flt-1. This may be attributed to binding of the excess recombinant protein to endogenous VEGF family ligands.
[4] Recovery was variable across cell culture supernatants; results will vary upon treatment conditions.
*VEGF-C was tested using N = 3 for serum and EDTA plasma, and N = 2 for cell culture supernatants.

Specificity

To assess assay specificity, the panel was tested under various conditions.

Each assay in the panel was tested individually by running a single calibrator with single detection antibodies. Non-specific interactions were less than 0.5% for all assays when running single calibrators with single detection antibodies.

Each assay in the panel was also tested using a blended calibrator and single detection antibodies. Non-specific binding levels were less than 0.5% for most assays in the kits. There were a few conditions that reported slightly higher non-specific binding levels when testing blended calibrators in the (1) Flt-1 assay with only VEGF-A detection antibody and (2) the VEGF-A assay with only the Flt-1 detection antibody. VEGF-A and VEGF-C are ligands for the Flt-1 receptor. Hence, receptor-ligand interactions may be contributing to the slightly higher levels of non-specific binding. The levels of non-specific binding were typically found to be below 5% and always observed to be below 10%.

Specificity was also tested for each assay with plasma matrices using individual detectors; less than 0.5% of non-specific interactions were observed in the native samples for all assays except VEGF-A and Flt-1.

Nonspecific binding was also evaluated for additional recombinant human proteins, including Ang-1, Ang-2, Ang-4, VEGFR-2, MMP-2, MMP-9, GRO-α, NRP-1, NRP-2, VEGF-B, Flt-4, and Angptl1, at concentrations of 0, 10, 100, and 1,000 ng/mL. Non-specific binding was <0.6% for all proteins except for Ang-1 and Ang-2 with the Tie-2 detection antibody where nonspecific binding was >20%. Since Ang-1 and Ang-2 are strong ligands for Tie-2, receptor interaction is to be expected.

Non-specificity reported in the COA for this panel is measured using blended calibrators and individual detection antibodies.

$$\%\ nonspecificity = \frac{nonspecific\ signal}{specific\ signal} \times 100$$

Species Cross-Reactivity

VEGF-A, VEGF-C, VEGF-D, Tie-2, Flt-1, PlGF, and FGF (basic) assays cross-react with non-human primate plasma samples.

VEGF-C and FGF (basic) assays cross-react with mouse serum samples.

Stability

The calibrator, controls, and diluents were tested for freeze-thaw stability. Results (not shown) demonstrated that the calibrator can tolerate one freeze-thaw cycle and controls can tolerate three freeze-thaw cycles without significantly affecting the performance of the assay. Diluents 7 and 11 are stable through two freeze-thaw cycles. Upon the first thaw, aliquot Diluent 7 and Diluent 11 into suitable volumes before refreezing. Once thawed, calibrator, controls, and diluents are stable for one week at 2–8ºC. Partially used MSD plates may be sealed and stored for up to 30 days at 2–8 °C in the original foil pouch with desiccant. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

Calibration

All the assays in the panel are calibrated against a reference calibrator generated at MSD.

MSD reference calibrators for the following analytes were evaluated against the NIBSC/WHO International Standards; the ratios of International Units of biological activity per mL (IU/mL) of NIBSC standard relative to pg/mL of MSD calibrator are shown in the table below. To convert MSD concentrations to biological activity relative to the WHO International Standard, multiply the MSD concentration by the ratio provided.

Table 9. Ratios of International Units (IU/mL) relative to MSD Calibrators (pg/mL)

Analyte	NIBSC/WHO Catalog Number	NIBSC (IU/mL): MSD (pg/mL)
VEGF-A	02/286	0.00068
FGF (basic)	90/712	0.0014

Tested Samples

Normal human samples and non-human primate (cynomolgus monkey) samples were diluted 2-fold and tested in the Angiogenesis Panel 1 (human) kit. Results for each sample set are displayed below. Concentrations are corrected for sample dilution.

Table 10. Normal human samples tested in the Angiogenesis Panel 1 (human) Kit

Sample Type	Statistic	VEGF-A	VEGF-C*	VEGF-D	Tie-2	Flt-1	PlGF	FGF (basic)[1]
Serum (N = 40)	Median (pg/mL)	194	537	925	6,153	126	6.31	1.76
	Range (pg/mL)	19.3–1,834	428–691	330–5,200	2,350–12,094	76.6–281	3.23–10.8	0.30–24.3
	% Detected	100	100	100	100	100	100	100
EDTA Plasma (N = 46)	Median (pg/mL)	88.7	205	812	6,198	82.9	5.77	1.19
	Range (pg/mL)	11.0–552	107–370	315–5,102	2,745–12,603	54.9–174	2.78–10.5	0.27–5.60
	% Detected	100	100	100	100	100	100	100
Heparin Plasma (N = 20)	Median (pg/mL)	76.8	144	956	7,430	70.1	6.65	N/A
	Range (pg/mL)	28.4–317	33.9–564	474–3,838	5,594–9,585	49.8–95.9	4.57–11.2	N/A
	% Detected	100	100	100	100	100	100	N/A
Urine (N = 10)	Median (pg/mL)	443	ND	19.6	15	11.6	27	0.69
	Range (pg/mL)	52.4–1,738	ND–16.1	ND–19.6	ND–15.7	1.63–44.7	7.09–40.9	0.15–9.09
	% Detected	100	20	10	20	100	100	100
CSF (N = 8)	Median (pg/mL)	7.06	30	52.5	70.7	38.8	32.9	0.45
	Range (pg/mL)	2.79–16.7	ND–44	24.4–140	25.3–253	30.2–172	6.83–71.1	0.29–1.11
	% Detected	100	73	100	100	100	100	100
Cell Culture Supernatants (N = 6)	Median (pg/mL)	1,048	84.1	19.4	236	7.76	665	74
	Range (pg/mL)	2.45–10,120	ND–300	ND–80.7	ND–457	ND–2,245	5.51–8,730	0.99–7,241
	% Detected	100	83	67	50	83	100	100
Cell Lysates (N = 7)	Median (pg/mL)	168	70.7	21.4	263	254	287	3,154
	Range (pg/mL)	2.83–3,219	40.1–1,245	ND–26.6	ND–76,416	3.65–4,212	10.0–1,750	1.35–8,393
	% Detected	100	100	57	86	100	100	100

[1]Heparin plasma is not a recommended matrix for FGF (basic) measurement
Median = median of detectable samples
% detected = % of samples with concentrations at or above the LLOD
ND = not detectable (below the LLOD)
*VEGF-C was tested using N = 5 for serum, EDTA plasma, heparin plasma, urine, and cell lysate, and N = 11 for CSF

Table 11. Non-human primate (cynomolgus monkey) samples tested in the Angiogenesis Panel 1 (human) Kit

Sample Type	Statistic	VEGF-A	VEGF-C	VEGF-D	Tie-2	Flt-1	PlGF	FGF (basic)
EDTA Plasma (N = 5)	Median (pg/mL)	11.6	6.6	353	126	63.7	2.88	4.57
	Range (pg/mL)	5.24–24.4	ND–16	305–537	ND–173	54.9–84.9	1.69–3.49	2.67–12.3
	% Detected	100	67	100	80	100	100	100

Median = median of detectable samples
% detected = % of samples with concentrations at or above the LLOD
ND = not detectable (below the LLOD)

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

Table 12. Recombinant human proteins used in the Calibrators

Calibrator	Expression System
VEGF-A	Insect cell line
VEGF-C	Mouse cell line
VEGF-D	Insect cell line
Tie-2	Mouse cell line
Flt-1	Insect cell line
PlGF	*E. coli*
FGF (basic)	*E. coli*

Antibodies

Table 13. Antibody source species

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
VEGF-A	Mouse Monoclonal	Mouse Monoclonal	C
VEGF-C	Mouse Monoclonal	Mouse Monoclonal	B
VEGF-D	Mouse Monoclonal	Goat Polyclonal	A
Tie-2	Mouse Monoclonal	Goat Polyclonal	A
Flt-1	Mouse Monoclonal	Goat Polyclonal	A
PlGF	Mouse Monoclonal	Mouse Monoclonal	B
FGF (basic)	Mouse Monoclonal	Mouse Monoclonal	A

References

1. Lee JW, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006;23:312-28.
2. Bowen RA, et al. Impact of blood collection devices on clinical chemistry assays. Clin Biochem. 2010;43:4-25.

Appendix A

The calibration curves below compare results for each assay in the 7-plex panel when the plate is run as a multiplex using all seven detection antibodies (graphs in blue; Blended Detectors) vs. running each assay using a single, assay-specific detection antibody (graphs in red; Single Detector).

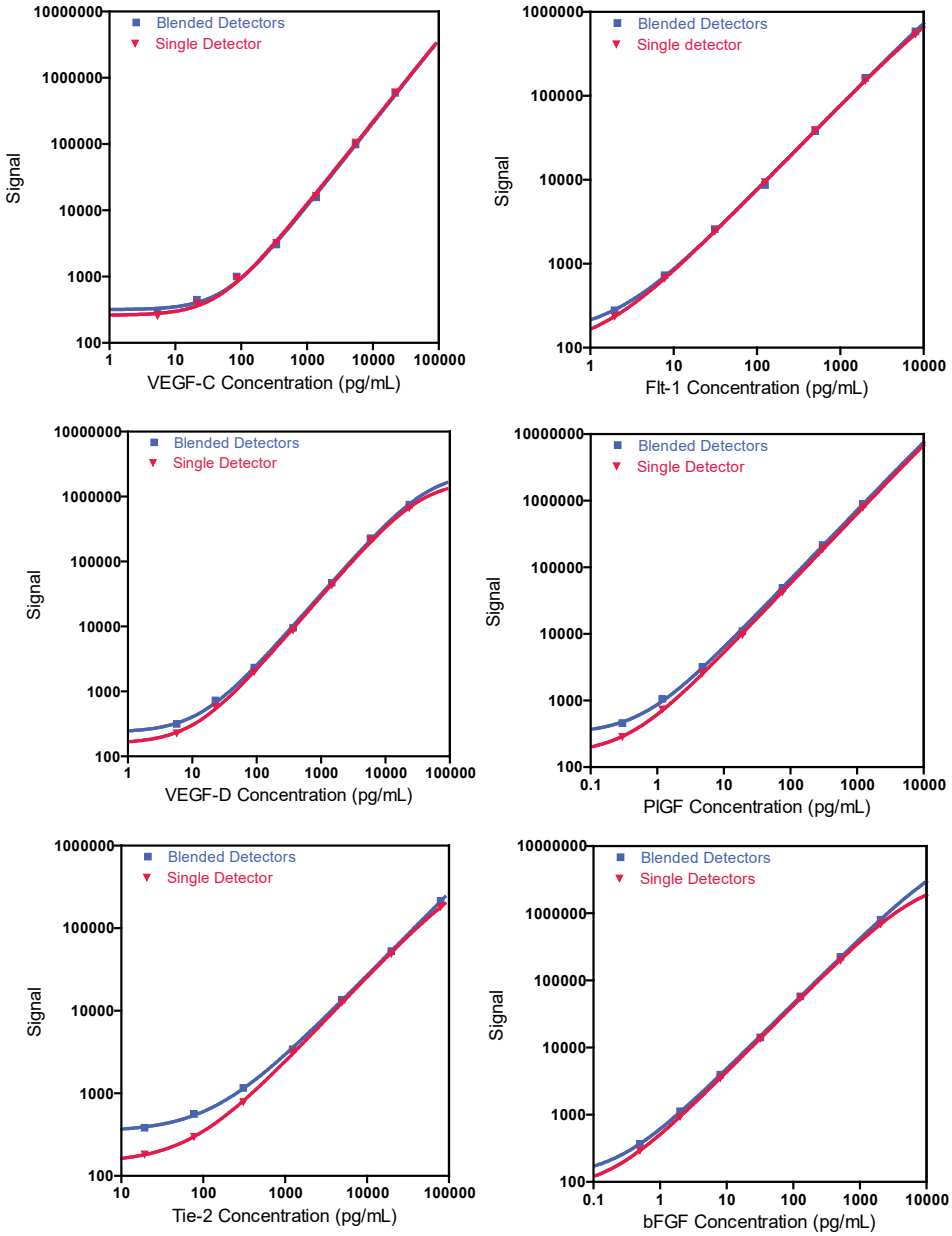

Table 14. *LLODs for detection of a single antibody vs. blended antibodies.*

	LLOD (pg/mL)	
Assay	7-spot plate, 1 Ab	7-plex, blended Abs
VEGF-C	4.51	10.5
VEGF-D	2.39	2.22
Tie-2	14.1	25.0
Flt-1	0.50	0.53
PlGF	0.08	0.10
FGF (basic)	0.08	0.07

As expected, both multiplex formats yielded the same specific signal, but lower background signals were seen when using the single detection antibody.

Summary Protocol

Angiogenesis Panel 1 (human) Kits

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol before performing the Angiogenesis Panel 1 (human) Assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature, thawing calibrator and controls on ice for at least 30 minutes.
- ❑ Prepare Blocker A solution.
- ❑ Prepare calibration solutions in Diluent 7 using the supplied calibrator:
  - o Dilute the stock calibrator 20-fold in Diluent 7.
  - o Perform a series of 4-fold dilution steps and prepare a zero calibrator.
- ❑ Dilute samples and controls 2-fold in Diluent 7 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 11.
- ❑ Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.

STEP 1: Add Blocker A Solution

- ❑ Add 150 µL/well of Blocker A Solution.
- ❑ Incubate at room temperature with shaking for 1 hour.

STEP 2: Wash and Add Sample

- ❑ Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 50 µL/well of sample (calibrators, controls, or unknowns).
- ❑ Incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Add Detection Antibody Solution

- ❑ Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 25 µL/well of 1X detection antibody solution.
- ❑ Incubate at room temperature with shaking for 2 hours.

STEP 4: Wash and Read Plate

- ❑ Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 150 µL/well of 2X Read Buffer T.
- ❑ Analyze plate on the MSD instrument.

Catalog Numbers

Table 15. Catalog numbers for V-PLEX and V-PLEX Plus angiogenesis (human) multiplex and single assay kits

Kit Name	V-PLEX			V-PLEX Plus*		
	1-Plate Kit	5-Plate Kit	25-Plate Kit	1-Plate Kit	5-Plate Kit	25-Plate Kit
Multiplex Assay Kits						
Angiogenesis Panel 1 (human)	K15190D-1	K15190D-2	K15190D-4	K15190G-1	K15190G-2	K15190G-4
Individual Assay Kits						
Human VEGF-C	K151LTD-1	K151LTD-2	K151LTD-4	K151LTG-1	K151LTG-2	K151LTG-4
Human VEGF-D	K151LUD-1	K151LUD-2	K151LUD-4	K151LUG-1	K151LUG-2	K151LUG-4
Human Tie-2	K151KWD-1	K151KWD-2	K151KWD-4	K151KWG-1	K151KWG-2	K151KWG-4
Human Flt-1	K151RZD-1	K151RZD-2	K151RZD-4	K151RZG-1	K151RZG-2	K151RZG-4
Human PlGF	K151MED-1	K151MED-2	K151MED-4	K151MEG-1	K151MEG-2	K151MEG-4
Human FGF (basic)	K151MDD-1	K151MDD-2	K151MDD-4	K151MDG-1	K151MDG-2	K151MDG-4

*V-PLEX Plus kits include controls, plate seals, and wash buffer. See **Kit Components** for details.

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL-01		Sample-01		Sample-09		Sample-17		Sample-25		Sample-33	
B	CAL-02		Sample-02		Sample-10		Sample-18		Sample-26		Sample-34	
C	CAL-03		Sample-03		Sample-11		Sample-19		Sample-27		Sample-35	
D	CAL-04		Sample-04		Sample-12		Sample-20		Sample-28		Sample-36	
E	CAL-05		Sample-05		Sample-13		Sample-21		Sample-29		Sample-37	
F	CAL-06		Sample-06		Sample-14		Sample-22		Sample-30		Sample-38	
G	CAL-07		Sample-07		Sample-15		Sample-23		Sample-31		Sample-39	
H	CAL-08		Sample-08		Sample-16		Sample-24		Sample-32		Sample-40	

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL-01		Control 1.1		Sample-06		Sample-14		Sample-22		Sample-30	
B	CAL-02		Control 1.2		Sample-07		Sample-15		Sample-23		Sample-31	
C	CAL-03		Control 1.3		Sample-08		Sample-16		Sample-24		Sample-32	
D	CAL-04		Sample-01		Sample-09		Sample-17		Sample-25		Sample-33	
E	CAL-05		Sample-02		Sample-10		Sample-18		Sample-26		Sample-34	
F	CAL-06		Sample-03		Sample-11		Sample-19		Sample-27		Sample-35	
G	CAL-07		Sample-04		Sample-12		Sample-20		Sample-28		Sample-36	
H	CAL-08		Sample-05		Sample-13		Sample-21		Sample-29		Sample-37	

Figure 5. Sample plate layout that can be used for the assays. Each sample, calibrator, and control (Plus Kit) is measured in duplicate in side-by-side wells.

Plate Diagram

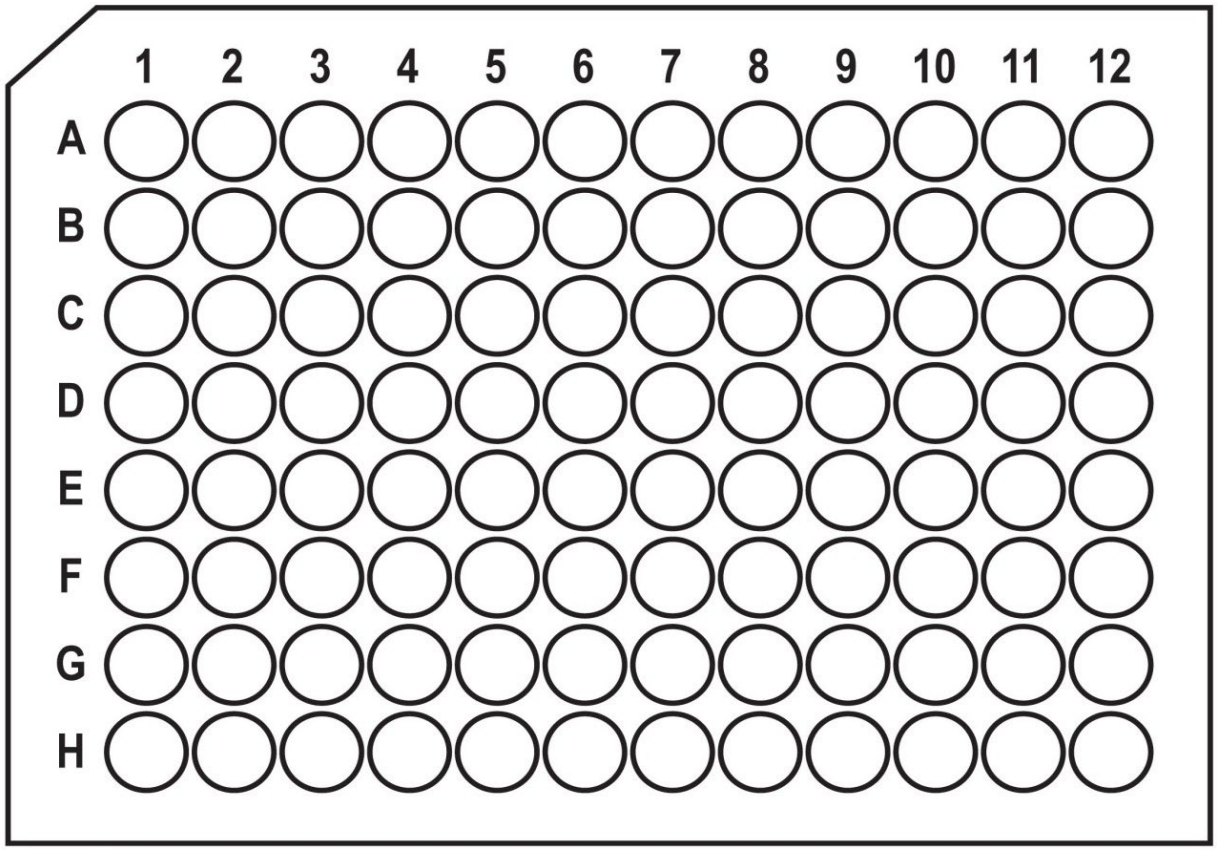

Figure 6. Plate diagram; a similar plate diagram can be created in Excel and easily impmorted into DISCOVERY WORKBENCH® software.